The role of microRNA genes in papillary thyroid carcinoma

Huiling He*, Krystian Jazdzewski*, Wei Li*, Sandya Liyanarachchi*, Rebecca Nagy*, Stefano Volinia*, George A. Calin*, Chang-gong Liu*, Kaarle Franssila†, Saul Suster‡, Richard T. Kloos§, Carlo M. Croce*, and Albert de la Chapelle*¶

*Human Cancer Genetics Program, ‡Department of Pathology, and §Departments of Internal Medicine and Radiology, Comprehensive Cancer Center, Ohio State University, Columbus, OH 43210; and †Department of Pathology, Helsinki University Central Hospital, FIN-00029, Helsinki, Finland

Contributed by Albert de la Chapelle, November 9, 2005

Apart from alterations in the RET/PTC-RAS-BRAF pathway, comparatively little is known about the genetics of papillary thyroid carcinoma (PTC). We show that numerous microRNAs (miRNAs) are transcriptionally up-regulated in PTC tumors compared with unaffected thyroid tissue. A set of five miRNAs, including the three most up-regulated ones (miR-221, -222, and -146), distinguished unequivocally between PTC and normal thyroid. Additionally, miR-221 was up-regulated in unaffected thyroid tissue in several PTC patients, presumably an early event in carcinogenesis. Tumors in which the up-regulation (11- to 19-fold) of miR-221, -222, and -146 was strongest showed dramatic loss of *KIT* transcript and Kit protein. In 5 of 10 such cases, this down expression was associated with germline single-nucleotide changes in the two recognition sequences in *KIT* for these miRNAs. We conclude that up-regulation of several miRs and regulation of *KIT* are involved in PTC pathogenesis, and that sequence changes in genes targeted by miRNAs can contribute to their regulation.

gene expression | KIT | DNA polymorphism

Papillary thyroid carcinoma (PTC) is the most common malignancy in thyroid tissue, accounting for ≈80% of all thyroid cancers. The incidence of PTC in the United States has increased in recent years (1). Genetically, PTC is characterized by alterations in the RET/PTC-RAS-BRAF signaling pathway (2, 3). Activating mutations in *BRAF* and *RET/PTC* gene rearrangements are frequent genetic changes in PTC tumors (4–6). A strong inherited genetic predisposition is suggested by case-control studies showing a 3- to 8-fold risk in first-degree relatives, one of the highest of all cancers (7, 8). Despite unequivocal evidence of an inherited predisposition, large families displaying Mendelian inheritance of PTC are rare, and no predisposing gene mutations have been found, even though several putative loci have been identified by linkage analysis (9–11).

MicroRNAs (miRNAs) represent a previously uncharacterized class of gene products that are believed to function as negative regulators of gene expression (12–16). Recently, miRNA genes have been implicated in several cancers (14, 17–25). The expression of miRNAs varies between cancer and normal cells and varies among different types of cancer. In most cancers studied so far, the expression of miRNAs seems to be lower than in the corresponding normal tissue (17, 18, 21, 22, 24).

We reasoned that the previous failure to identify genes predisposing or contributing to PTC might be because these genes show low penetrance. The mechanisms may require the interaction of two or more genes; thus, regulatory, rather than protein-encoding, genes might be involved. We undertook this study to elucidate the role of regulatory genes such as the miRNAs in the predisposition and development of PTC.

Methods

Patient and Control Samples and Cell Lines: Nucleic Acid Extraction. After approval of the Institutional Review Board and patient consent, fresh samples from the tumor tissue PTC (T-PTC) and normal thyroid tissue adjacent to PTC tumors (N-PTC) were obtained from 20 patients with sporadic PTC undergoing surgical resection. The samples were snap-frozen in liquid nitrogen and stored at −80°C. Clinical data and information on the specimens are shown in Table 2, which is published as supporting information on the PNAS web site. Normal thyroid tissue (N-Thy, $n = 6$) was collected from consenting individuals who had surgery because of laryngeal malignancy but no thyroid disease. Other samples included: paraffin blocks of thyroid tissue from Finnish sporadic PTC patients ($n = 135$); blood DNA samples of random Finnish control individuals ($n = 100$); lymphoblastoid cell DNA samples ($n = 24$) from Centre d'Etude du Polymorphisme Humain control individuals who are not genetically related. Thyroid cancer cell lines K1, K2, and NPA87 were cultured in DMEM mixed with F12 and MCDB (ratio 2:1:1) medium supplemented with 10% FBS and glutamine in a 5% CO_2 incubator. Genomic DNA and RNA were extracted from the samples by standard techniques. Total RNA was extracted with TRIzol solution (Invitrogen), and the integrity of RNA was assessed by using an Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA).

miRNA Microarrays. Biotin-labeled miRNA was hybridized on miRNA chips as described in ref. 26. Briefly, 5 μg of total RNA from each sample was reverse transcribed by using biotin end-labeled random octamers. Hybridization was carried out on our new custom miRNA microarray chip (OSU_CCC version 2.0), which contained 460 mature miRNA probes spotted in quadruplicate (235 *Homo sapiens*, 222 *Mus musculus*, and 3 *Arabidopsis thaliana*) with annotated active sites. The hybridized chips were washed and processed to detect biotin-containing transcripts by streptavidin-Alexa647 conjugate and scanned by using an Axon 4000B (Axon Instruments). Scanned images were quantified by GENEPIX PRO 6.0 (Axon Instruments).

Gene Expression Arrays. Affymetrix HG-U133 plus two chips (Affymetrix, Santa Clara, CA) were used to evaluate genome-wide gene expression levels. The experimental procedure was according to the Affymetrix Gene-Chip Expression Analysis manual. Double-stranded cDNA was synthesized by using the Superscript Choice System, followed by an *in vitro* transcription reaction with a T-7 (dT24) primer to produce biotinylated cRNA. The full-length cRNAs were fragmented to 20 to 200 bp and hybridized to Affymetrix GeneChip Human Genome U133 Plus 2.0 Array.

Conflict of interest statement: No conflicts declared.

Abbreviations: miRNA, microRNA; PTC, papillary thyroid carcinoma; N-PTC, normal thyroid tissue adjacent to PTC tumors; T-PTC, tumor tissue PTC.

Data deposition: Gene expression data with Affymetrix chips were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (NCBI GEO accession no. GSE3467). MicroRNA chip data have been deposited at the ArrayExpress database (accession no. E-TABM-68).

¶To whom correspondence should be addressed. E-mail: albert.delachapelle@osumc.edu.

Statistical and Bioinformatics Analysis of Microarray Data. Raw data from miRNA chips were assembled by using BRB ArrayTools (Richard Simon and Amy Peng Lam, National Cancer Institute, Bethesda). BRB ArrayTools generated an average value of the four spot replicates for each miRNA. Background subtracted intensities were thresholded to 10 and log transformed. Flagged spots corresponding to absent or low-quality signals were removed from the analysis before global median normalization. Significant analysis of microarrays and prediction analysis of microarrays were performed to identify differentially expressed miRNAs in PTC tumors and miRNAs for tumor classification, respectively. Data from Affymetrix chips were analyzed by using the robust multichip average to calculate normalized expression values. Significance analysis of microarrays and permutation tests were used to find differentially expressed genes.

Northern Blot Analysis of miRNA. Northern blotting was performed as described in ref. 17. Ten micrograms of total RNA from snap-frozen tissues and cell lines was loaded onto a precast 15% denaturing polyacrylamide gel (Bio-Rad). The RNA was then electrophoretically transferred to Bright-Star blotting membranes (Ambian). The oligonucleotides used as probes were the complementary sequences of the mature miRNA (miRNA Registry): miR-221, 5′-GAAACCCAGCAGACAATGTAGCT-3′; miR-146b, 5′-AGCCTATGGAATTCAGTTCTCA-3′. Probes were end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (USB, Cleveland). Prehybridization and hybridization were carried out in Ultrahyb Oligo solution (Ambian) containing 10^6 cpm/ml probes overnight at 42°C. The most stringent wash was with 2× SSC and 1% SDS at 42°C. For reuse, blots were stripped by boiling and reprobed. As a loading control, 5S rRNA was visualized by ethidium bromide staining of the gel before transfer. The image of Northern hybridization signals was produced by using STORMSCANNER and IMAGEQUANT TL software (Molecular Dynamics).

Semiquantitative and Quantitative RT-PCR. Total RNA was first treated with DNase-1 (Ambian) and then reverse transcribed to cDNA with the SuperScript First Strand Synthesis system (Invitrogen). Target genes and an endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were included in the same PCR reaction for semiquantitative RT-PCR. All PCR reactions were verified to be in the linear range by testing with different cycle numbers. Quantitative real-time PCR was performed by using an ABI PRISM 7700 DNA Sequence Detection System (Applied Biosystems) and a SYBR Green PCR kit (Applied Biosystems). The comparative threshold cycle method was used to calculate the relative gene expression. Primers for amplification of *GAPDH*, miR-221, miR-222, miR-146a, miR-146b, and *CITED1* are listed in Table 3, which is published as supporting information on the PNAS web site.

Western Blot. Total protein extract was prepared by using lysis buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, plus proteinase inhibitors. The protein concentration was determined by the Bio-Rad protein assay. Thirty micrograms of each protein lysate was loaded onto SDS/PAGE under reducing conditions and transferred to poly(vinylidene difluoride) membrane (Millipore). The membrane was incubated in 5% nonfat dry milk in T-TBS (18 mM Tris·HCl, pH 7.6/122 mM NaCl/0.1% Tween 20) at room temperature for 2 h followed by incubation with a monoclonal anti-Kit antibody (BD Biosciences) and a secondary antibody of horseradish peroxidase-conjugated goat anti-mouse IgG. Immune complexes were detected with the Supersignal Western Pico Chemiluminescent Substrate (Pierce). Loading control was done by reincubating the same membrane with an anti-GAPDH antibody (Novus Biological).

Table 1. Differentially expressed miRNAs in PTC tumors

MiRNA	Fold	Localfdr, %
hsa-mir-146	19.3	4.4
hsa-mir-221	12.3	3.2
hsa-mir-222*	10.9	2.7
hsa-mir-21*	4.3	6.7
hsa-mir-220	4.0	8.6
hsa-mir-181a	2.6	6.9
hsa-mir-181c	2.4	7.6
hsa-mir-181*	2.2	7.2
hsa-mir-155	2.2	9.1
hsa-mir-213	1.9	7.3
hsa-mir-34a	1.8	21.9
hsa-mir-24-2	1.7	10.1
hsa-mir-29a-2	1.7	21.7
hsa-mir-29b	1.7	23.7
hsa-mir-29c	1.6	21.6
hsa-mir-102	1.6	11.2
hsa-mir-24-1	1.5	14.5
hsa-mir-9-3	0.7	7.3
hsa-mir-219-1	0.6	5.7
hsa-mir-138-1	0.6	5.0
hsa-mir-138-2	0.6	4.9
hsa-mir-345	0.6	4.7
hsa-mir-26a-1	0.6	5.0

A summary of SAM analysis of paired T-PTC and unaffected thyroid tissue from 15 PTC patients. Mean of fold changes; Localfdr, local false discovery rates. *q* value (%) of all miRNAs was 0.

*The chips had two probes of mir-222, two probes of mir-21, and three probes of mir-181b. The different probes showed no significant fold variation.

Sequencing. DNA specimens were amplified by using a standard PCR protocol. The PCR products were purified with ExoSAP-IT purification kit (USB) and sequenced in both directions with the ABI sequencing system (PerkinElmer Applied Biosystems). The sequencing results were analyzed by using DNAStar SEQMAN software. The PCR primers used for *KIT* exons and miRNA sequencing are listed in Table 3.

Hybridization Prediction. The minimum free energy hybridization of miRNAs and target mRNA with all polymorphic variants were predicted by RNAHYBRID software (27).

Results

Distinct Overexpression of miRNAs in PTC Tumor Tissues. Initially miRNA expression was analyzed in paired PTC tumor and unaffected thyroid tissue from nine patients. Pairwise significance analysis of microarray analysis indicated that six miRNA genes were significantly overexpressed in T-PTC with fold changes >1.5 in at least seven patients. These miRNAs were as follows: miR-146, miR-221, miR-222, miR-21, miR-155, and miR-181a (Table 1). The top three miRNAs (miR-146, miR-221, and miR-222) showed dramatic overexpression, with 11- to 19-fold higher level in PTC tumors compared to the adjacent unaffected thyroid tissue. We repeated the miRNA chip experiments with samples from another six PTC patients. The overexpression of the six miRNAs identified previously was fully confirmed in these new samples. Data analysis of the combined 30 samples from 15 PTC patients indicated that the upregulation of miRNAs in PTC tumors represented a distinct miRNA signature comprising 23 miRNAs. Among the significant differentially expressed miRNA genes, overexpression occurred in 17. Underexpression occurred in only six miRNAs, and in all of them, the change was <2-fold (Table 1).

To identify the smallest set of predictive miRNAs distinguish-

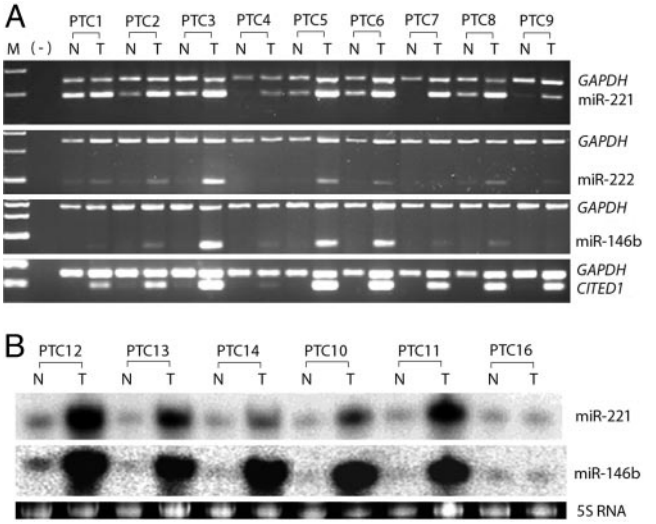

Fig. 1. MiRNA expression microarray data confirmation. (*A*) Semiquantitative RT-PCR of miR-221, miR-222, miR-146b, and *CITED1* in the paired PTC samples from nine patients. *GAPDH* was used as an internal control for RT-PCR; M, DNA marker; (−) negative control; N, normal thyroid tissue; T, tumor tissue. (*B*) Northern blot showing the overexpression of mature miR-221 and miR-146b in PTC tumors. The same filter was used for both hybridizations after stripping. 5S RNA in an ethidium bromide gel was used as loading control.

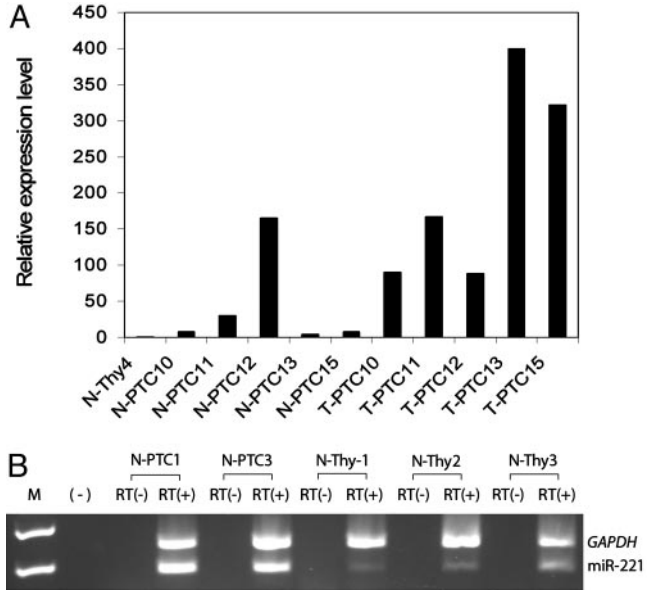

Fig. 2. Overexpression of miR-221 in normal thyroid tissue (N-PTC) adjacent to PTC tumor (T-PTC). (*A*) Real-time RT-PCR quantification of miR-221 expression in N-PTCs and T-PTCs. The expression level of sample N-Thy4 (thyroid tissue from an individual without thyroid disease) was set as a reference (N-Thy4 expression = 1). (*B*) Semiquantitative RT-PCR of miR-221 expression in two N-PTC samples and three samples of normal thyroid tissue from individuals without clinical thyroid disease (N-Thy). RT (−) and RT (+), absence vs. presence of reverse transcriptase in the RT-PCR reactions.

ing between normal and cancer tissue, we performed class prediction analysis by using the first set (18 samples) to predict the outcome of the second set (12 samples). Results from the prediction analysis of microarrays analysis indicated that only five overexpressed miRNAs (miR-221, miR-222, miR-146, miR-21, and miR-181a) were sufficient to successfully predict cancer status. No classification errors were made in this analysis (Fig. 5, which is published as supporting information on the PNAS web site).

Confirmation of Overexpression of miR-221, miR-222, and miR-146b in PTC Tumors. We performed RT-PCR and Northern blot analysis of the top three overexpressed miRNAs in PTC tissue samples and in thyroid tumor cell lines and normal thyroid tissue samples. Overexpression of miR-221 and miR-222 in PTC tumors was confirmed by both semiquantitative RT-PCR and Northern blot (Fig. 1 *A* and *B*). The expression patterns of miR-221 and miR-222 in PTC tumors were concordant in most of the cases. miR-221 and miR-222 are located close to each other on the X chromosome. The concordant expression patterns in different PTC samples suggest shared regulatory mechanisms for the expression of these two clustered miRNAs.

The probe sequence of miR-146 on the chip was designed corresponding to miR-146a, located on chromosome 5. Recently miR-146b on chromosome 10 was identified. The mature forms of these two miRNAs have high sequence homology, only two nucleotides being different. Using primers specific for miR-146a and miR-146b in their premature forms, no visible RT-PCR band of miR-146a was detectable in thyroid tissue. In contrast, miR-146b was significantly overexpressed in PTC tumor samples (Fig. 1*A*). This result indicated that miR-146a either was not expressed in thyroid tissue or the expression level was very low. Thus the overexpressed miR-146 on the microchips apparently reflects miR-146b. Indeed, the mature form of miR-146b was strongly overexpressed in PTC tumors as confirmed by Northern blot (Fig. 1*B*).

Overexpression of miR-221 in N-PTC. The expression of miR-221 was detectable in normal thyroid tissue adjacent to PTC tumors (N-PTC) from all patients (Fig. 1*A*). Interestingly, the miR-221 expression level in at least two N-PTC samples was much higher than in the rest of them. For example, there was a strong band of miR-221 in samples N-PTC1 and N-PTC3 shown by semiquantitative RT-PCR (Fig. 1*A*). To examine whether this observation might be an artifact due to tumor tissue contaminating the "normal" tissue, we used the same samples to demonstrate the expression of *CITED1*, a molecule that is known to be highly expressed in PTC tumors but not in normal thyroid (28). Expression of the *CITED1* gene was not detectable in the same set of RNA samples of these normal thyroid tissues, but overexpressed in the corresponding paired tumors (Fig. 1*A*) excluding significant contamination. The variation of miR-221 in normal thyroid tissues was further tested with other N-PTC samples and compared to normal thyroid tissue from individuals without any clinical thyroid disease. Quantitative real-time PCR revealed the same observation in five sample pairs (Fig. 2*A*). The overexpressed miR-221 in two N-PTC samples was not due to contamination of the RNA preparation with genomic DNA (Fig. 2*B*). Considered together, our data suggest that increased expression of miR-221 in normal thyroid tissue might be an early genetic event in PTC carcinogenesis.

Small Percentage of Putative miRNA Target Genes Showing Down-Expression at the mRNA Level in PTC Tumors. miRNAs interact with their target genes and, thereby, play regulatory roles in many physiological functions and pathophysiological processes. We analyzed the predicted targets of the three most significantly overexpressed miRNAs (221, 222, and 146). The analysis was performed by using three publicly available algorithms to predict human miRNA gene targets, i.e., MIRANDA (29), TARGETSCAN (30), and PICTAR (31). To reduce the number of false positives, we listed only putative target genes predicted by at least two of the programs (Table 4, which is published as supporting information on the PNAS web site). The above approach produced 130 putative targets of miR-221. Although *KIT* was only pre-

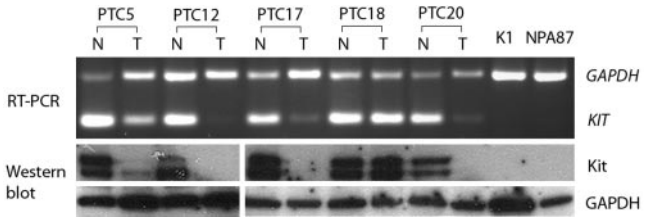

Fig. 3. Down-expression of *KIT* transcript and Kit protein in PTC tumor tissues. Semiquantitative RT-PCR of *KIT* expression in five paired PTC samples and two PTC cell lines. Western blot of Kit in protein extract from the same sample set. Bands of ≈140 kDa and ≈120 kDa represent the mature and immature forms of Kit protein, respectively. GAPDH was used as a loading control.

dicted by MIRANDA, we included *KIT* in this list, because *KIT* is a target of miR-221 as demonstrated by biological methods (32).

It is believed that miRNAs interact with target genes at specific sites by inducing cleavage of the targeted message or by inhibiting translation (12). Therefore, one might predict that an overexpressed miRNA would be associated with down-expression of its targets at the transcript or protein level. We examined the gene expression level of the putative targets of miR-221, -222, and -146 in the same set of RNA samples. This analysis was done first by evaluating genomewide gene expression levels in PTC tumors and matched normal thyroid tissues by using the Affymetrix chip. Significance analysis of microarray analysis revealed a list of genes differentially expressed (unpublished data). Most of these genes showed expression behaviors consistent with our previously published data (28). For example, overexpression of the *CITED1* gene was found in all PTC tumors tested and was confirmed by semiquantitative RT-PCR (Fig. 1*A*). Comparing the lists of putative miRNA targets (as defined above) and differentially expressed genes, we found that the differentially expressed mRNAs accounted only for <15% of the total target genes (data not shown). Further analysis of miR-221 targets was performed by k-means clustering of the relative expression changes between tumor/normal pairs of target genes (using gene expression ratios) and miRNAs (using inverse miRNA expression ratios) by using the EPCLUST program (http://ep.ebi.ac.uk/EP/EPCLUST). It appeared that a group of target genes (84 probe sets) that showed down-expression in PTC tumors was clustered together with miR-221 and miR-222. Within this cluster, 19 genes were identified whose expression level was significantly down in PTC tumors compared to their paired unaffected tissue (random variance *t* test *P* value <0.005; permutation test *P* value <0.05; fold changes ≥1.5). The expression patterns of miR-221, miR-222, and these 19 targets are illustrated in Fig. 6, which is published as supporting information on the PNAS web site. *KIT* was one of the genes showing profound down-expression of mRNA in PTC tumors.

Down-Expression of KIT Transcript and Protein in PTC Tumors. Western blotting of Kit protein was performed with protein extracts obtained from five patients and two cell lines (Fig. 3). Hybridization with a monoclonal anti-Kit antibody revealed two bands of ≈140 kDa and 120 kDa, respectively, corresponding to the mature fully glycosylated and the partially glycosylated Kit (33) (Fig. 3). Both forms of Kit were readily detected in normal thyroid tissue but were strongly reduced or absent in the four samples showing strong reduction of *KIT* transcript. The down-expression of *KIT* transcript and Kit protein level correlated well with the strong overexpression of miRs-221, -222, and -146b in these four PTC patient samples (Fig. 1). However, in two further cases with *KIT* transcript down-regulation, Kit protein was unchanged (data not shown).

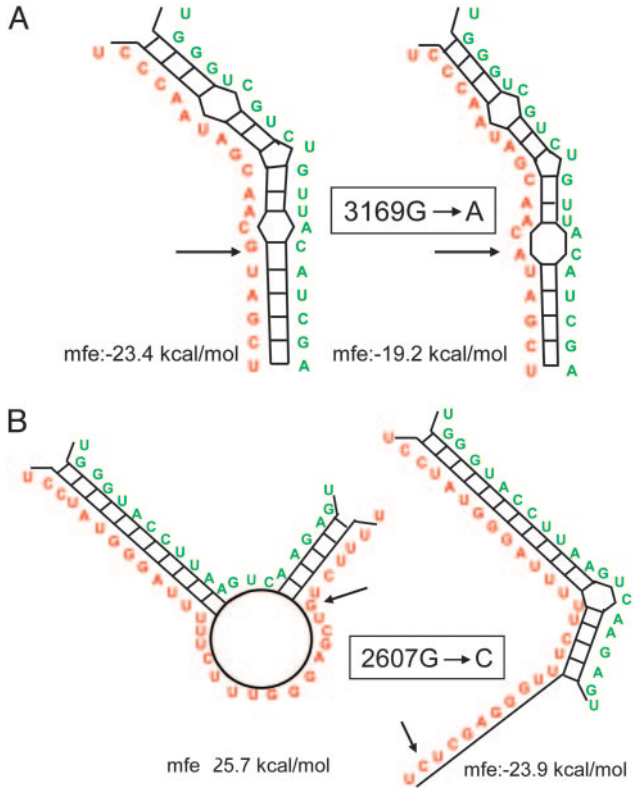

Fig. 4. Computational modeling of the interaction of miR-221 and miR-146 with the *KIT* gene. (*A*) Hybridization of miR-221 (green color) and *KIT* mRNA (red color); arrows highlight a polymorphic site within the binding domain (SNP rs17084733). mfe, minimum free energy. (*B*) Hybridization of miR-146b (green color) and *KIT* mRNA (red color); arrows highlight a polymorphic site within the binding domain (SNP rs3733542).

Mutations in the *KIT* Gene. We sequenced the miR-221, -222, and -146b genes and their flanking regions in samples from 48 PTC patients and three PTC cell lines. No previously unidentified sequence changes or DNA polymorphisms were found (data not shown).

miRNA-binding domains in the *KIT* sequence were predicted with several software programs (27, 29, 31). We sequenced the regions harboring two binding domains, one for miR-221 and miR-222, and another one for miR-146a and miR-146b, in genomic DNA samples from the PTC patients. We found a polymorphism in each recognition site. The 3169G→A SNP (rs17084733) is located within the crucial region of the miR-221 and miR-222 domain in the *KIT* 3′ UTR region. We found GA heterozygosity in five PTC patients. Heterozygosity for 3169G→A leads to a conformation change with an increase of free energy (Fig. 4*A*). The synonymous 2607G→C SNP (rs3733542) in exon 18 is located within the crucial region of the miR-146a and miR-146b domain. This genotype leads to changes in miRNA:target gene mRNA duplex conformation and results in hybridization with a different region (Fig. 4*B*). We found GC heterozygosity in the same five PTC patients, suggesting linkage disequilibrium. Notably, the tumors of all these five doubly heterozygous patients (PTC3, PTC6, PTC8, PTC10, and PTC11) had up-regulation of the three top miRs and profound down-regulation of the *KIT* transcript. Sequencing of all of the remaining *KIT* exons in nine PTC cases and three cell lines disclosed one additional, previously unreported change in exon 17 (ex17 + 33, C>T) in one PTC patient. To exclude that the rare alleles at these SNPs themselves predispose to PTC, we compared the allele frequencies in cohorts of sporadic PTC

patients and healthy controls (both cohorts from Finland). No association was found (Table 5, which is published as supporting information on the PNAS web site).

Discussion

A relatively unexpected finding of ours was the highly preferential increase rather than decrease in transcript of many miRNA genes in the tumors compared with paired unaffected thyroid tissue. Such up-regulation has previously been noted in a few cancers, but decreases in the tumor seem to be more common (21, 22, 24). For example, miR-15 and miR-16 are frequently down-regulated in B cell chronic lymphocytic leukemia (17); miR-143 and miR-145 showed decreased expression in colorectal tumors (34); miRNA let-7 was down-expressed in human lung cancer, which is associated with poor prognosis (24). Down-expression of miRNAs was also found in cancers of the breast, kidney, prostate, and uterus (21, 22). Over-expression of miRNAs was reported in some tumors: miR-155 was overexpressed in Burkitt's lymphomas and human B cell lymphoma (35), and a cluster of miRNAs, the miR-17–92 polycistron, were overexpressed in B cell lymphoma patients (23). Although we have no clear explanation of the preferential up- rather than down-regulation of miRNAs in PTC, we note that this cancer has unusually little loss of heterozygosity (LOH) (36, 37). If LOH is generally associated with loss of mRNA transcript, then the paucity of LOH might, in part, explain our findings. It follows that genomic amplification might explain abundant miRNA transcript.

Comparatively little is known about genetic changes in PTC, and no predisposing mutations have been described. Our findings are compatible with the hypothesis that RNA-based regulatory mechanisms involving miRNAs, and possibly other RNA genes, characterize this tumor. The existence of regulatory networks, including inherited changes therein, is now established in principle, although almost nothing is known about the genes themselves (38).

We observed that miR-221 expression was not only up-regulated in PTC tumors, but also showed clear-cut variation in the unaffected thyroid tissue among patients with PTC. In at least 3 of 15 patients, the up-regulation was strong. This observation suggests that unaffected normal thyroid tissue adjacent to tumors may harbor genetic changes before the appearance of morphological malignancy. Overexpression of miR-221 could be a premalignant change in PTC. Taken together, miRNA deregulation in the thyroid could be a critical component of PTC initiation and development. We hypothesize that miR-221 may function as an oncogene in the thyroid. Future study is warranted to explore the significance of the overexpression of miRNAs in thyroid tissues from PTC patients.

Kit is an important tyrosine kinase receptor in cell differentiation and growth; it functions as an oncogene in many cancers (39, 40). The transcript level of *KIT* in PTC tumor cells is known to be extremely low (41, 42), which is consistent with our data. In contrast, reports about the protein level in PTC are contradictory (43, 44). Although in several PTC tumors we found Kit protein to be down-regulated, this observation did not apply to all tumors, emphasizing the complexity of these regulatory pathways, which may well be organ- or cell-specific.

Computational predictions and experimental approaches support the idea that different miRNAs target the same mRNA (29). Multiple miRNAs have been predicted to target *KIT*, including those overexpressed in PTC (27, 29, 31). We show here that in most PTCs at least three miRs (221, 222, and 146) targeting *KIT* were up-regulated. It is entirely possible that the multiple interaction opportunities provided by networks or "signatures" of miRNA dysregulation create different responses in target genes under different circumstances and combinations.

Perhaps most interestingly, our results highlight the role of the target genes themselves in their responses to miRNA interaction. We found a SNP in *KIT* precisely in the crucial region of the 3′ UTR-binding domain for miR-221 and -222, and another SNP precisely in the crucial region of the exonic binding domain for miR-146a and -146b. The crucial region is a stretch of seven nucleotides in the miRNA:mRNA duplex, usually located in the 5′ end of the miRNA, starting at the first or second position. It is likely that variants within the binding regions can acquire new miRNAs as regulating factors due to sequence changes such as SNPs. Remarkably, of 10 tumors in which the key miRNA genes were up-regulated, all 10 showed low or very low *KIT* transcript, and 5 of these patients were the only ones (of 15 tested) showing heterozygosity for the germline SNPs. These findings suggest that not only changes in miRNAs but also in their target genes (inherited or perhaps acquired) profoundly influence PTC carcinogenesis. Our association study suggests that these events are downstream of the putative genetic initiating or predisposing events. More detailed functional studies must address questions such as how miR-221, -222, and -146b interact with their binding domains in the *KIT* gene, the effects of the two SNPs on the interactions, and the combinational effects of miR-221, -222, and -146b on *KIT* and other possible targets.

We thank Jan Lockman and Heejei Yoon for help and advice. This work was supported by National Institutes of Health (NIH) Grants CA16058 (to A.d.l.C.) and P01 CA78890 (to C.M.C.). BRB ArrayTools was developed by Dr. Richard Simon and Amy Peng Lam. Thyroid tissue samples were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute.

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